MAPPING THE GENOMIC BINDING SITES OF THE ACTIVATED RETINOID X RECEPTOR IN MURINE BONE MARROW DERIVED MACROPHAGES USING CHROMATIN IMMUNOPRECIPITATION-SEQUENCING

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***RunningTitle**: ChIPing the cistrome of RXR.*

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Summary

Chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-Seq) is a powerful technique to map the genomic location of a given chromatin bound factor (i.e., transcription factors, co-factors) or epigenetic marks, such as histone modification. The procedure is based on, crosslinking of proteins to DNA followed by the capture of the protein-DNA complexes by "ChIP-grade" antibodies. In this chapter we describe in detail the experimental method developed in our laboratory to investigate *in vivo* the DNA binding characteristics of a key heterodimeric nuclear receptor, the Retinoid X Receptor (RXR) in murine bone marrow derived macrophages.

Keywords: *Macrophage**;** chromatin**;** binding site**;** ChIP**;** RXR**;** cistrome.*

1. Introduction

Nuclear hormone receptors are lipid activated transcription factors that regulate gene expression in a ligand dependent manner. They possess an evolutionarily conserved domain structure, which consists of a DNA binding domain responsible for the recognition of a specific motif encoded in the genome, a ligand binding domain for binding the activator ligand and the transactivation domain for transcription initiation. Ligand binding leads to conformation changes on the surface of the receptor, followed by a co-repressor - co-activator switch/exchange, which then results in a distinct change in gene transcription (reviewed in *1*).

RXR is an essential member of the nuclear receptor family, because it forms heterodimers with other nuclear hormone receptors (Liver X Receptor, LXR; Peroxisome Proliferator Activated Receptor, PPAR; Retinoic Acid Receptor, RAR etc.) that regulate indispensable biological processes such as inflammation, lipid and glucose metabolism *(2, 3)*. RXR was discovered as a novel retinoid responsive transcription factor *(4)*. Several ligands, such as 9-cis retinoic acid, docosahexanoic acid and phytanic acid have been shown to activate the receptor, although their action as endogenous ligands under physiological conditions have not been proven yet *(5)*. There are also specific and selective synthetic agonists of RXR, such as LG100268 or LG10069 (Bexarotene) *(6, 7)*.

RXR has three different isotypes in metazoans (RXRα, RXRβ, RXRγ), which display differential expression patterns in different tissues. For example, RXRα is expressed in heart, liver, kidney, spleen and placenta epidermis, RXRγ is specific to brain and muscle, while RXRβ is ubiquitous *(5)*. The phenotypes attributable to these isoforms have been well characterized. For instance, abolished expression of RXRα was shown to be lethal at embryonic stage E13.5-16.5 mostly due to cardiac abnormalities, while the lack of RXRβ or RXRγ was not fatal, but resulted in male infertility and increased metabolic rate. These

studies also revealed a locomotor deficiency in RXRβ/γ double *knock out* mice *(8, 9)*. In cells of myeloid origin RXRα appears to be the dominant isoform *(10, 11)*.

On the other hand, activation of RXR and the molecular details of RXR mediated gene expression remains enigmatic, because: 1. Definitve proof for a biological role for an endogenous ligand is still lacking. 2. It is not clear if it also has heterodimer independent activities.

ChIP-Seq has become the most widely used and effective method to study chromatin state and transcription factor distribution at the genomic level. ChIP was almost the first application linked to next generation sequencing and the one leading to the determination of the typical histone acetylation and methylation patterns of gene promoters, enhancers, insulators and repressed chromatin territories *(12)*. ChIP-Seq also allows the determination of the "cistrome" of any transcription factor meaning - all the binding sites in a given cell type under given circumstances, which was first carried out for Signal Transducer and Activator of Transcription (STAT) 1 *(13)*. Macrophages are a major target of research including studies on immune function, but also in metabolism and transcriptional regulation as well *(14, 15)*. This is not only because their relatively easy accessibility, but also due to their importance in physiological as well as pathological processes.

In order to better understand the molecular mechanisms by which RXR regulates gene expression in a genome-wide manner in murine bone marrow derived macrophages, a protocol was developed in our laboratory to accurately map the receptor binding sites using ChIP-Seq. This protocol is described in this chapter **(Figure 1)**.

The protocol here described is optimized to murine bone marrow derived macrophages. A similar approach was used by us to determine the RXR cistrome in HeLa cells *(16)*. Optimization to other cell types would be required especially regarding cross-linking and sonication.

2. Materials

1. Bone marrow derived macrophages obtained from the femur of C57Bl6/J male animals as described *(17)*. Briefly, bone-marrow was flushed, cells were purified through a Ficoll-Paque gradient and cultured in DMEM containing 20% endotoxin-reduced fetal bovine serum and 30% L929 conditioned medium for 5 days. On the sixth day DMEM is replaced to Macrophage serum free media for 24 hours, then treatments are performed.
2. DSG Di (N-succinimidyl) glutarate (see **Note 1**).
3. DMSO (dimethyl-sulfoxide).
4. DMSO/EtOH (1:1) as vehicle treatment.
5. LG268 (Ligand Pharmaceuticals) dissolved in DMSO/EtOH
6. Ultrapure formaldehyde 16% (Thermo Scientific, PI-28906) (see **Note 1**).
7. 1M Glycine.
8. PBS (Phosphate Buffered Saline).
9. Roche Complete Mini Protease Inhibitor Tablets (EDTA-free).
10. Cell Lysis/ Wash Buffer: 0.15M NaCl, 0.005M EDTA pH 7.5, 0.05M Tris-HCl pH 7.5, 0.5% NP40, dH_2O supplemented with protease inhibitor (Roche) prior to use. Store at 4· C.
11. 1 ml Insulin syringe.
12. Shearing Buffer 0.05M Tris-HCl, pH 8.0, 1% SDS, 0.01M EDTA, dH_2O supplemented with protease inhibitor tablets (Roche) prior to use. Store at room temperature. (see **Note 4**)

13. Dilution Buffer: 0.001M EDTA, pH 8.0, 0.01% SDS, 1.1% Triton-X 100, 0.17M NaCl, dH_2O supplemented with protease inhibitor tablets (Roche) prior to use. Store at 4°C.
14. 0.5% Bovine Serum Albumin (BSA)/ PBS. Filter through 0.22 μm filter using a syringe. Prepare freshly before use.
15. IgG (Millipore)
16. ChIP-grade antibody to RXR (Santa Cruz Biotechnology).
17. 15 ml conical tubes (polystyrene) (see **Note 6**).
18. 100% Ethanol (ETOH).
19. Agilent 2100 Bioanalyzer with 7500 chips for inputs, 1000 chips for DNA libraries.
20. Axygen LoBind tubes (1.5ml)
21. Dynabeads® Protein A (10002D)
22. PBS containing 0.5% BSA (filter through a 0.22 μm filter using syringe)
23. Magnetic Rack for 1.5ml eppendorf tubes and for 15ml conical tubes
24. TE buffer: 10mM Tris HCl pH 8.0, 1mM EDTA, dH_2O (without protease inhibitor). Store at 4°C.
25. Bead Elution Buffer: 0.1M $NaHCO_3$, 1% SDS, dH_2O (make fresh immediately before elution)
26. RNase A 10μg/μl.
27. Proteinase K 20 μg/μl.
28. 3M Sodium acetate, pH 5.2.
29. Qiagen MinElute PCR Purification Kit.
30. Sonicator, Diagenode Bioruptor® standard (Cat. No. UCD-200).
31. Thermomixer.
32. Rotating Tube Rack or platform in cold room.

33. Ovation Ultralow Library Systems (Nugen).

3. Methods

Prepare the lysis, dilution and shearing buffers by dissolving the protease inhibitor tablets according to the manufacturer's instructions. Make sure that you have sufficient amount of PBS at room temperature and at 4°C (20 ml ice-cold PBS for washing/ plate, 20 ml PBS at room temperature for cross-linking/ plate).

3.1 Crosslink and harvest the cells

1. Approximately $3x10^7$ bone marrow derived macrophages are used as starting material for transcription factor ChIP-Seq. About 10^7 cells are seeded per plate (15 cm diameter) and the chromatin obtained from three plates is combined during the experiment for each reaction.
2. Treat the cells with vehicle (DMSO/EtOH) or 100 nM LG268 for 1 hour or more, depending on the given experiment.
3. Dissolve DSG in DMSO (50mg DSG is dissolved in 300 µl DMSO yielding a 0.5M DSG solution (see **Note 1**).
4. Dilute DSG to 0.002M in PBS, at room temperature.
5. Remove the medium from the cells and pipette 10 ml of DSG containing PBS onto the plates.
6. Incubate for 30 minutes at room temperature. Gently swirl the plates every 5 minutes.
7. During the last 5 minutes, prepare 1% formaldehyde solution in PBS at room temperature (see **Note 1**).
8. After incubation, aspirate the DSG solution and replace it with 10 ml 1% formaldehyde solution. Incubate for 10 minutes at room temperature. Gently swirl the plates every 2 minutes. Make sure that the DSG solution is completely discarded before adding the formaldehyde solution (see **Note 2**).

9. Add 1.5 ml of 1M glycine directly to the formaldehyde containing solution and incubate the cells at room temperature for an additional 5 minutes, while gently swirl the plate in every minute.
10. Place the samples on ice.
11. Aspirate the liquid from the plates and wash the cells twice with ice-cold PBS. Make sure that after the second wash all the supernatant is discarded, then immediately proceed to lysis.

3.2 Cell lysis and chromatin shearing

1. Scrape up the cells from each plate in 1 ml Cell Lysis/Wash Buffer containing appropriate amount of protease inhibitors (see **Note 3**).
2. Transfer the lysates to 1.5 ml centrifuge tubes.
3. Centrifuge with 12.000 x g for one minute at 4°C.
4. Remove the supernatant and add 1 ml of Cell Lysis/ Wash Buffer.
5. Pipette up and down the cells at least 20 times to get homogenous cell suspension, and then push through the whole volume in a 1 ml insulin syringe.
6. Repeat the step 3.
7. Remove the supernatants and combine the pellets into one centrifuge tube, using the same 1 ml Lysis Buffer. Push the whole volume again through a 1 ml insulin syringe.
8. Repeat the step 3.
9. Remove the supernatant and resuspend the nuclear pellet in 1 ml of room temperature Shearing Buffer (see **Note 4**).
10. Gently pipette up and down at least 30 times to generate a homogenous suspension and take care not to generate bubbles (see **Note 5**).
11. Transfer the solutions into 15 ml conical tubes (polystyrene) (see **Note 6**) and place the sonicator probes into the tubes (see **Note 7**)

12. Sonicate the chromatin to get fragments between 200-500 bp. Using the Diagenode Bioruptor® Standard model, 3 x 5 minute long cycles are used, with 30 seconds on, 30 seconds off set-up. The first sonication cycle is carried out at high and the other two at low power setting (3 cycles, 15 minutes) (see **Note 8** and **Note 9**).
13. Transfer the sheared chromatin to 1.5ml tubes and centrifuge at 12.000x g for 10 minutes at 4°C. Set aside 20 µl of sheared chromatin as input to check the fragment size distribution and normalize quantitative PCR measurements (see **Note 10**).
14. Transfer 900 µl supernatant to a new 15 ml conical tube and dilute 10 fold by adding 8.1 ml Dilution Buffer containing protease inhibitors. The diluted chromatin can be stored for 24 hours at 4°C.

3.3 Immunoprecipitation

1. Add 5 µg of anti-RXR antibody to each diluted chromatin.
2. Set aside at least 1 ml of diluted chromatin and add 5 µg of pre-immune rabbit IgG, as a control of nonspecific binding.
3. Incubate the samples overnight at 4°C, using an 'end-over-end' rotator.
4. On the day of immunoprecipitation, pre-block the paramagnetic beads. For each immunoprecipitation, prepare 190 µl of beads. Wash the beads three times with 1 ml of PBS containing 0.5% BSA, using the magnetic rack appropriate for 1.5 ml centrifuge tubes. Incubate the beads overnight at 4°C using rotator (see **Note 11**).
5. After overnight incubation, centrifuge the chromatin antibody complexes at 3500 rpm for 20 minutes at 4°C.
6. Use the top 90% of the centrifuged chromatin for bead binding (see **Note 12**).
7. Use the magnetic rack to collect the beads and replace the supernatant with 190 µl of PBS containing 0.5% BSA.

8. Combine the beads with the antibody chromatin complexes and incubate for at least four hours at 4°C using the rotator (see **Note 13**).
9. Prepare appropriate amount of Cell Lysis/ Wash Buffer by adding 1 protease inhibitor tablet to 50 ml of Cell Lysis/ Wash Buffer.
10. Place the tubes containing the antibody-chromatin-bead complexes on a magnetic rack at 4°C.
11. Incubate for 2 min or until the liquid appears clear, and then aspirate the supernatant.
12. Add the same volume of Cell Lysis/ Wash Buffer as the immunoprecipitation volume (RXR-8 ml, IgG-1 ml) and rotate the tubes for 3 minutes at 4°C.
13. Wash (six times) the complexes with Cell Lysis/ Wash Buffer. Carefully remove all traces of buffer at the end of the last washing step, without disturbing the beads and place the tubes on ice.
14. Add 1 ml ice-cold TE Buffer. Gently pipette up and down to generate homogenous bead slurry then transfer the whole volume to a 1.5 ml LoBind tube.
15. Place the LoBind tubes on a magnetic rack, which stands on ice. Wait until the liquid appears clear then remove the supernatant (see **Note 14**).

3.4 Bead elution, de-crosslinking and DNA purification

The remaining part of the protocol should be carried out at room temperature.

1. Add 100 µl Bead elution buffer to each tube and vortex at moderate speed for a few seconds. Place the samples on a thermomixer for 15 minutes and shake at 1000 rpm.
2. Place the tubes back on the magnetic rack and collect the supernatant into clean LoBind tubes.
3. Repeat the step 1.

4. After the second elution step, add 8 µl of 5M NaCl to each sample and incubate overnight at 65°C to de-crosslink the immunoprecipitated chromatin. Process the input DNA in the same way for QPCR measurements.
5. On the following day, add 1 µl of 10 µg/ µl RNase A to each sample and incubate for 30 minutes at 37°C.
6. Add 8 µl of 1M Tris-HCl, pH 8.0, 4 µl of 0.5M EDTA and 1 µl of 20 µg/ ml Proteinase K and incubate for at least 2 hours at 45°C on a thermomixer at 1000 rpm.
7. Purify the immunoprecipitated DNA (total volume 222 µl) using Qiagen MinElute columns, according to manufacturer's instruction.
8. Add 1110 µl PB Buffer to each sample and acidify the solution by adding 50 µl of 3M sodium acetate (see **Note 15**).
9. Elute the immunoprecipitated DNA in 15 µl Elution Buffer.
10. Proceed to library preparation and/or QPCR measurements.
11. For library preparation the Ovation Ultralow Library Systems is used. Libraries are generated from 5 ng of immunoprecipitated DNA, according to manufacturer's protocol (see **Note 16**).

4. Notes

1. Dissolve DSG immediately before use as it is highly unstable in solution and always use freshly opened formaldehyde ampulla for cross-linking. Otherwise, the cross-linking efficiency might be seriously affected.
2. Make sure that all the DSG is discarded before formaldehyde cross-linking, since DSG and formaldehyde cross-reaction might lead to quenched formaldehyde efficiency.
3. Scraping up the cells in PBS will cause the cells to stick to the wall of the centrifuge tubes during centrifugation that causes a subsequent loss in cell number.

4. Store and use Shearing Buffer at room temperature, because SDS precipitation occurs at 4°C. This negatively affects sonication efficiency. If SDS precipitation occurs in the nuclear lysate, allow the solution to come to room temperature before sonication.
5. Be careful not to generate bubbles during lysis, which can negatively affect sonication efficiency.
6. It is highly recommended to use polystyrene tubes, instead of polypropylene, since polystyrene transfers sonic waves more efficiently.
7. The probes should be set exactly in the middle of the tube submerged in the solution. If the probes come in contact with the inner surface of the tubes it might negatively affect sonication efficiency.
8. Check the position of the probes after each sonication cycle to make sure they not to touch the tube.
9. In order to determine the optimal sonication conditions for your cells perform a series of experiment with different power, cycle number, etc. Use the condition resulting in the highest enrichment of a positive control region after QPCR measurement, which typically gives the best sequencing results. For QPCR measurements of RXR ChIP, we used the following primers: Abca1 -78bp Fw: TGCCGCGACTAGTTCCTT, Abca1 -78bp Rev: TCTCCACGTGCTTTCTGCT, Abcg1 +1kb Fw: CCGAATCGTGCCTTTCTTT, Abcg1 +1kb Rev: GTGGGCTTCTAGCGTATTG. As a negative control region use: 36b4 Fw: GGGGAGATCCCAAGACTACAG, 36b4 Rev: CCACGCGCTTTAACAGAGTT
10. For input isolation, add 3 volumes of 100% EtOH to the 20 µl sheared chromatin, vortex and incubate overnight at -80°C. The next day, centrifuge it for 10 minutes at maximum speed (4°C). Remove the supernatant, dry the DNA pellets and dissolve in 200 µl Elution Buffer. Process input DNA from step 4 in section 3.4.

11. Avoid that magnetic beads remain in the bottom of the tubes, since it causes incomplete blocking and high background signal after sequencing. Therefore, transfer the tubes immediately from the magnetic rack to the rotator before the overnight blocking step.
12. Be careful not to disturb the chromatin-antibody complexes after centrifugation. Use only the top 90% of the sample to avoid high background signal.
13. To avoid incomplete mixing of the magnetic beads and the antibody-chromatin complexes see **Note 11** for instructions.
14. Use 1 ml pipette tips to remove all the supernatants at this step, because the beads are loosely attached to the tube surface.
15. Adsorption of DNA to the silica during column purification is drastically reduced at higher pH. It is important to follow the manufacturer's instructions to acidify the DNA solution prior to binding to the column.
16. To determine the background of the experiment prepare control libraries with the input DNA and DNA obtained from samples immunoprecipitated with control IgG.

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6. Figure Legend

Figure 1. Schematic representation of the main steps of the ChIP-Seq protocol.